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On the Morphogenesis of Feathers

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Abstract

The most unique character of the feather is its highly ordered hierarchical branched structure^{1, 2}. This evolutionary novelty confers flight function to birds^{3–5}. Recent discoveries of fossils in China have prompted keen interest in the origin and evolution of feathers^{6–14}. However, controversy arises whether the irregularly branched integumentary fibers on dinosaurs such as *Sinornithosaurus* are truly feathers^{6, 11}, and whether an integumentary appendage with a major central shaft and notched edges is a non-avian feather or a proto-feather^{8–10}. Here we take a developmental approach to analyze molecular mechanisms in feather branching morphogenesis. We have used the replication competent avian sarcoma (RCAS) retrovirus¹⁵ to efficiently deliver exogenous genes to regenerating chicken flight feather follicles. We show that the antagonistic balance between *noggin* and bone morphogenetic protein 4 (BMP4) plays a critical role in feather branching, with BMP4 promoting rachis formation and barb fusion, and *noggin* enhancing rachis and barb branching. Furthermore we show that sonic hedgehog (SHH) is essential for apoptosis of the marginal plate epithelia to become spaces between barbs. Our analyses show the molecular pathways underlying the topological transformation of feathers from cylindrical epithelia to the hierarchical branched structures, and provide first clues on the possible developmental mechanisms in the evolution of feather forms.

With three branching levels, i.e. from rachis to barbs; from barbs to barbules and from barbules to cilia or hooklets¹ (Fig. 1a), feathers can develop into a variety of forms, including the downy, contour, flight feathers, etc. (Fig. 1b). As in hairs, the feather follicle is composed of a dermal papilla and epidermal collar (equivalent to the hair matrix, Fig. 1c–f). Through epithelial-mesenchymal interactions, the epithelial cells at the bottom of the follicle undergo active proliferation (proliferation zone, Fig. 1c). Immediately above, the epithelial cells start to form the rachidial ridge and the barb ridges (ramogenic zone, Fig. 1c, f)^{16–19}. Further distal, the barb ridge epithelia actively proliferate and differentiate to form the marginal plates, barbule plates and axial plates (Fig. 1e, central part). The barb ridges grow to form barbs, composed of the ramus and barbules, while the marginal and axial plate cells die to become the intervening space. Individual barbule plate cells undergo further cell shape changes to form the cilia and hooklets¹. The barb ridges fused proximally to form the

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Competing interests statement

The authors declare that they have no competing financial interests.

rachidial ridge, which will become the rachis. Additional cross sections (Supplementary Information Fig. 1) illustrate this process.

The cellular and molecular mechanisms of epithelial organ morphogenesis are beginning to be understood^{20, 21}. While branching morphogenesis²¹ has been studied in the lung and kidney, branching in the feather is unique for its exquisite order and non-randomness. In this work, we studied the role of Noggin/BMP interactions that underlie fundamental morphogenetic mechanisms^{22–24}, in this process. We first analyzed the dynamic expressions of BMP2, BMP4 and Noggin in remiges (flight feathers) of 15-day old chicken embryos (E15) using in situ hybridization. BMP4 transcripts were detected in the dermal papilla and overlying pulp area (Fig. 1g). Later, BMP4 was expressed in the barbule plate cells (Fig. 1i, Supplementary Information Fig. 2). BMP2 was in the marginal plate epithelia in early ramogenesis²⁵, but quickly switched to barbule plate epithelia (Fig. 1i–k). BMP4 expression in the mesenchyme appeared to form a gradient, tapering from the proximal to distal regions (Fig. 1g). Noggin transcripts were detected in the pulp cells, overlapping with BMP4 transcripts. Noggin was not expressed in the dermal papilla, but was expressed in the pulp regions adjacent to the epidermis. The expression level of noggin appeared to form a gradient from the proximal to distal pulp, with highest expression at the level of the ramogenic zone (Fig. 1h). Cross sections at the indicated locations (dotted lines) are also shown (Supplementary Information Fig. 2).

A distinct feature of the feathers is that they can regenerate repetitively after plucking. Remige feathers regenerate at a rate of about 0.5 cm/day, making them excellent recipients for RCAS mediated gene expression, which only transduces cells undergoing active mitosis¹⁵. By injecting RCAS retroviral constructs into chick flight feather follicles after plucking (Fig. 2a), exogenous genes were mis-expressed during feather regeneration (Fig. 2b, Supplementary Information Fig. 3). Regenerated feathers injected with RCAS-LacZ virus showed no changes in the rachis, and barbs (Fig. 2c, g).

RCAS-noggin was injected into the feather follicles to perturb their BMP activity (Fig. 2d, h; n=36). Many of the regenerated feathers were severely stunted forming few barbs (33%) and even more had the rachis split into 2 or 4 mini-rachises (44%). These smaller rachises gradually converged at the proximal end (Fig. 2d). Barbs of the noggin expressing regenerated feathers were inhibited (50%) and some barbs (11%) further branched into two (Fig. 2h). Histological examination showed that the barb ridges formed irregular tree-like structures with extravagant ridge formations (Fig. 3b, f, j). The rachidial ridge was fragmented into smaller ridges (Fig. 3n).

Regenerated feathers expressing ectopic BMPs had phenotypes that were generally opposite to that of feathers expressing exogenous noggin. Many of the RCAS-BMP4 transduced regenerated feathers were short in length, did not form barbs or a rachis (50%), and assumed a morphology similar to the calamus (feather shaft) (n=16). Several of these regenerated feathers were oversized (25%). Ectopic rachis-like structures were observed (Fig 2e, 31%). The barbs were often fused (Fig. 2i; 38%). Histological sections showed that the barb ridges failed to separate (Fig. 3c, g), the barbs fused (Fig. 3k), and the rachidial ridges were

oversized (Fig. 3o). The presumptive marginal plate cells became plump (Fig. 3g) and did not die to become space (Fig. 3K).

BMP2 over-expression had similar phenotypic effects as BMP4. Feathers showed normal growth at first, then died abruptly and fell off prematurely in about 3 weeks. The sizes were usually smaller and some showed no barb formation. The rachises of the regenerated feathers were enlarged (Fig. 2f; 23%, n=26), and some feathers had ectopic rachises (23%). Some barbs fused with each other or with the rachis at various points, forming bundles (Fig. 2f, j, 77%). Histological sections showed that some barb ridges fused in pairs (Fig. 3h), suggesting that BMP2 may also function in specifying marginal plate fate, given that BMP2 was expressed transiently in peripheral marginal plates (Fig. 1j). Ectopic rachidial ridge-like structures caused by the fusion of barb ridges were observed (Fig. 3l). The rachidial ridge was extremely large, spanning nearly half of the follicle circumference (Fig. 3p). The phenotypes of regenerated feathers appeared to depend on the expression levels of the transduced genes.

While both abnormal barb ridges shared a forked appearance, the identity of barb branching or barb fusion was distinguished by counting the number and spacing of barbs. Control samples had about 6 barb ridges in the space shown in Fig. 3e–h. Noggin treated specimens had elaborately branched ridges alternating with mini-ridge forms, but the total number of ridges was unchanged. BMP2 treated specimens had only 3 forked barb ridges, suggesting that fusion occurred among the original 6 ridges.

Marginal plate cells expressed SHH²⁶ and barbule plate cells expressed feather keratin (Fig. 3q, u; 4a). Characterization of the transduced feathers showed that noggin increased branching by increasing the number of SHH positive marginal plate cells (Fig. 3r) and perturbed the shape and arrangement of the feather keratin expressing barbule plate cells (Fig. 3v). On the other hand, epithelial over-expression of BMPs 2 and 4 altered the fate of marginal plate cells. The cells, which were plump, rather than flattened (Fig. 3 g, k) did not die and branches failed to form. SHH was not expressed (Fig. 3s, t). Some feather keratinization still took place in the barbule plate (Fig. 3w, x).

To further test the role of SHH in feather branching, we suppressed SHH using cyclopamine²⁷ or RCAS–antisense SHH in the plucked and regenerating feather model. The two independent reagents gave similar results. The regenerated feathers showed regions where barbs fused with a web-like membrane between; therefore forming continuous feather vanes (Fig. 4b, c). Cross sections showed regions with barb ridges that failed to separate because the marginal plate cells failed to disappear (Fig. 4d, e). Suppressing SHH produced a similar phenotype as over-expressing BMP4 (compare Fig. 3g and 4e). In control feathers, TUNEL staining was positive in the marginal epithelial cells, pulp epithelium and feather sheath (Fig. 4f). The death of these cells allows feather branches to open. Suppression of SHH activity increased marginal plate cell numbers. These cells were plump in appearance and were mostly TUNEL negative (Fig. 4g). Thus, BMP over-expression suppressed SHH expression and the subsequent formation of the marginal plate. Suppression of BMP promoted branching, probably by enhancing ridge-forming activity of the basilar cells, together with specifying marginal plate fate. Therefore SHH is required for specifying

marginal plate fate. Balancing the antagonistic actions of SHH and BMPs sets the number and spacing of barb ridges. However, regulation of Shh by other molecules is also possible.

Recently, the importance of the SHH/BMP “signaling module”²⁸ in skin appendage morphogenesis was also shown by comparing chicken feather variants, duck feathers, and avian and alligator scales²⁵. Using embryonic chicken explant cultures, they suggest that SHH is important for proliferation in proximal barb ridges, while BMP 2 suppresses SHH and promotes differentiation of distal barb ridges²⁵. Our in vivo “transgenic feather” model using plucked/regenerated mature feather follicles allows us to analyze the late branching event that is impossible to observe in embryonic explants. This novel model should open doors for future experiments to link molecular pathways with feather forms.

Based on these data, we propose the following model for feather branching morphogenesis (Fig. 5a, b). The multilayered epidermis can be molded into epidermal ridges by ridge forming activators (e.g., noggin) and inhibitors (e.g., BMPs) distributed in the adjacent mesenchyme. The balance between noggin and BMP4 determines the number, size and spacing of barb ridges. 1) At the proliferation zone, the level of BMP4 is much higher than that of noggin, thus the epithelial cells form a cylindrical structure. 2) At the ramogenic zone, the level of noggin in the pulp adjacent to epithelia gradually exceeds that of BMP4, thus the epithelial cells start to form multiple barb ridges. 3) The basilar layer becomes periodically arranged into SHH positive/BMP2 transiently positive marginal plate cells that die, and SHH negative barb ridge growth zone cells that proliferate. The intermediate cell layer is “cleaved” into groups of cells that become the initial barb ridge. Marginal plate cells die to ensure the formation of spaces between barb ridges^{1, 29}. 4) The originally randomly arranged cells in the barb ridge express BMPs 2 and 4, line up and become two rows of barbule cells. These cells further differentiate to form the barbules. Barbule plate cells¹ stimulated by BMP2 and 4 change shape to form the cilia and hooklets. 5) Toward the end of feather formation, noggin activity is reduced and conditions revert back to (1), forming the calamus without branches at the proximal end of the feather shaft. 6) If the noggin/BMP ratio becomes polarized in the anterior- posterior axis, the site with higher BMP activity eventually becomes the rachis, thus the bilaterally symmetric feather can form.

In this work, we showed that the BMP pathway regulates the formation and inter-conversion of the barbs and rachis. We also showed that the true separation of branches required SHH activity. Other morphogens, such as FGFs and Wnts¹⁸, may also be involved in this process, and we expect them to behave under similar principles along the same pathway or with special regulation to produce feather variants.

Formation of hierarchical branches is the cardinal feature of feathers¹⁷, and therefore one of the key issues in the origin and evolution of feathers. Based on some fossil evidence, it has been proposed that a filamentous integument structure with a major central shaft and notched edges may be the prototype of feathers^{8–10}. According to this model, the rachis would have formed first in evolution, then barbs, and finally barbules. Therefore, the rachis and barbs would be different entities and not interchangeable (Fig. 5c). Alternatively, because barbs form first during development, it was proposed that barbs appeared first in integument evolution, and the rachis, a specialized form of fused barbs, appeared later as an

evolutionary novelty^{16, 18}. The fact that the barbs and rachis can be converted experimentally in the laboratory favors the Barb – Rachis model. The data here suggest that a radially symmetric feather is more primitive than the bilaterally symmetric feather in terms of molecular and developmental mechanisms, and may have been the prototype of feathers (Fig. 5c). Some fossilized primitive skin appendages on *Sinornithosaurus* also favor this model¹¹. Further modulation of BMP and SHH pathways may have led to the many feather varieties seen today by regulating the number, shape and size of the rachis, barbs, and barbules^{1, 17, 30}. This work provides the first evidence for the molecular mechanisms possibly involved in the evolution of feather branching.

Methods

Materials

Specific-pathogen-free fertilized white leghorn chicken eggs were purchased from Charles River SPAFAS (North Franklin, Connecticut). The eggs were incubated at 38°C in a humidified rotating incubator. Chicks were housed in the USC Vivarium. RCAS-noggin plasmid was from Dr. R. Johnson. RCAS-BMP2 and RCAS-BMP4 plasmids were from Dr. P. Francis-West. RCAS-LacZ plasmid encoding β -galactosidase was originally constructed by Dr. L. Yi and provided by Dr. W.-P. Wang. RCAS-Shh sense was from Dr. Tabin. RCAS-SHH antisense was produced by cutting the sense SHH plasmid with ClaI and religating the inserted SHH in the reverse orientation. The orientation was confirmed by restriction enzyme analysis (Ting-Berreth and Chuong, unpublished). Viruses were prepared and titrated²⁴. Cyclopamine, a SHH antagonist²⁶ provided by Dr. W. Gaffield of the USDA, was dissolved in absolute ethanol and diluted to 0.4 mg/ml in DMEM for injection (20 μ l) into the regenerating feathers, using a similar procedure described for viral transduction.

Transduction of regenerating feather follicles

Chickens at 2–3 weeks of age were anesthetized with Ketamine (50 mg/kg body weight). Primary remiges I–VII were used because of their large size, distinct morphology and identity. Normally, feathers will regenerate and grow out of the follicles at about 14 days after plucking. Regenerated feathers/feather follicles were dissected at 7 days after plucking. Serial longitudinal and cross paraffin sections were cut and stained with H & E. For gene transduction, RCAS viruses were injected into the empty follicles immediately after feathers were plucked. RCAS virus propagation in follicles was verified using RCAS-LacZ virus followed by X-gal staining of cryostat sections at various days after the injection. Each follicle received about 10–20 μ l of medium containing the virus (1×10^5 – 1×10^6 infectious units/ml). For preliminary studies, viruses containing the genes of interest were injected into primary remige follicles on the left wing. RCAS-LacZ virus was injected into primary remige follicles on the right wing as controls. Feather follicles injected with viruses were dissected and sectioned for histological study at 2–3 weeks after virus injection. Once distinct phenotypes were confirmed in at least 3 repeat experiments, viruses were injected to follicles on both wings for later studies. Chickens were raised in cages and observed on a daily basis over a 2-month period. The regenerated feathers were plucked and examined with a dissection or scanning EM microscope for abnormalities compared with normal primary remiges.

Histology and in situ hybridization (ISH)

Paraffin sections (5 μ m) were stained with H&E or prepared for ISH following routine procedures²⁶. Cryostat sections (10 μ m) were stained with X-gal. TUNEL staining was performed using a kit (Roche). Nonradioactive ISH or section ISH was performed according to the protocol described^{22, 26}. Digoxigenin labeled probes are generated by in vitro transcription from plasmids kindly provided by Dr. Niswander for Noggin, Dr. Francis-West for BMP2, BMP4, and Dr. Johnson for noggin and Dr. Tabin for Shh. A probe for feather keratin was prepared in our laboratory. After hybridization, sections were incubated with an anti-digoxigenin Fab conjugated to alkaline phosphatase (Boehringer Mannheim). Color was detected by incubating with a BM purple substrate (Boehringer Mannheim).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Feather branching morphogenesis and gene expression. **a**, Diagram showing the three branching levels. Level I: Rachis (blue) branches into barbs (red). Ia, radially and Ib, bilaterally symmetric feathers. Level II: Barbs branch into barbules (green); Level III: Barbules branch into cilia and hooklets (purple). **b**, Different chicken feather types. **c**, Feather follicle structure schematic. **d–f**, BMP4, Noggin, and BMP2 expression patterns. The two dotted lines indicate the level of cross sections shown in Supplementary Information Fig. 2. **g**, Schematic drawing of feather barb. **h, i**, BMP2 in barb ridges. First in peripheral marginal plates (mp), then switch to barbule plates (bp). dp, dermal papilla; rz, ramogenic zone. Bar size, 100 μ m.

Figure 2. Phenotypic changes in feathers regenerated from follicles injected with RCAS-Noggin, BMP4, and BMP2 respectively

a, Diagram showing the gene expression strategy. **b**, X-gal staining of a regenerating feather follicle injected with RCAS-LacZ 7 days ago. **c–f**, Splitting or merging of the rachis is indicated by red arrows. **g–j**, Alteration of the barbs. Abnormal branch points are indicated by blue arrows. **k**, Diagram illustrating the overall phenotypic changes. Bar size, 50 μm (**c–f**, **i, j**), 100 μm (**g, h**).

Figure 3. Analyses of feathers injected with RCAS-Noggin, -BMP4, and -BMP2 respectively
a–d, Cross sections. **e–l**, Changes in barb ridges. **m–p**, Changes of rachidial ridge size (H&E). Rachis width (green arc). **p**, Enlarged indicated area (see inset) is part of the rachidial structure, not barb ridges (compare with Fig. 3e). **q–t**, ISH with SHH probes. Marginal plates or comparable regions (red arrows). **u–x**, ISH with feather keratin probes. Green lines delineate barb ridges. Dotted lines indicate abnormal barb ridges. rr, rachidial ridge. mp, marginal plate. bp, barbule plate. ap, axial plate. gz, barb ridge growth zone. Bar size, 100 μ m.

Figure 4. SHH roles in barb formation

a, Normal feather whole mount ISH of SHH and BMP2. **b**, SHH inhibition by RCAS-antisense Shh or cyclopamine produced fused vanes. **c**, Schematic depicting changes in **b**. Barbs (red). Barbules (green). **d**, Cross sections (H&E) showed barb ridge segments that failed to separate varying from more severe (5–9 o'clock region) to less severe (1–2 o'clock). **e**, Enlarged indicated area in **d**. **f**, **g**, Apoptosis in late-differentiated barb ridges in normal and SHH suppressed follicles. bp, barbule plate; mp, marginal plate; fs, feather sheath; pe, pulp epithelium. Bar size, 100µm.

Figure 5. Models of feather branching and evolution of feather forms

a, Roles of *Noggin*/*BMP4*, *Shh*, *BMP2* in the 3 levels of feather branching. **b**, The ratio of *noggin* and *BMP4* may determine the number and size of barb ridges. A localized high *BMP*/*noggin* ratio, together with a helical growth mode of barb ridges¹⁷, can lead to the formation of a rachidial ridge through fusion of barb ridges. **c**, Hypothetical models of the evolution of feather forms. Upper row, **Barb → Rachis model**. Lower row, **Rachis → Barb model**. The experimental data are in favor of the **Barb → Rachis model**.